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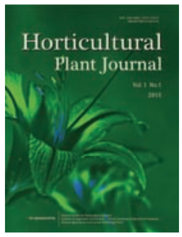

Effect of Bag Removing with Reflective Film Mulching Before Harvest on Fruit Coloration and Expression of Anthocyanin Related Genes in Peach

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Abstract

Appearance quality is the main element forming the fruit quality. Both bagging and reflective film application can improve appearance comprehensive quality. In order to determine the effects of bagging and reflective film on peach fruit coloration, this study explored the effects of applying reflective film before harvest on fruit appearance quality of peach (*Prunus persica*), and discussed the influencing mechanism on fruit coloration. Yellow outside and black inside double-layer bags were removed from fruit twelve days d before harvest. Peel color, pigment content, enzyme activity as well as the gene expression related to fruit coloration of a well-colored and late-ripening peach cultivar 'Xiahui 8' with different treatments (non-bagging with non-reflective film mulching, debagging with non-reflective film mulching, debagging with reflective film mulching) were researched. The results showed that both debagging with non-reflective film mulching and debagging with reflective film mulching could significantly improve red and green color difference value (a^*), a^*/b^* (b^* is yellow and blue color difference value), chroma (C), anthocyanin (Ant) content and reduce hue angle (h), while debagging with reflective film mulching had higher Ant content than debagging with non-reflective film mulching ($P < 0.05$). The two treatments up-regulated the transcript level of anthocyanin biosynthetic related genes (*UFGT*, *CHS*) at the beginning of the experiment, however, the expression of *DFR*, *LDOX* and *F3H* were enhanced along with the testing process. A downward trend on peel phenylalanine ammonia lyase (PAL) activity of non-bagging with non-reflective film mulching was observed during the experiment, however, the peel PAL activity of the other two treatments first increased and then decreased. What's more, peel PAL activity of debagging with reflective film mulching was higher than debagging with non-reflective film mulching. These results suggested that Ant synthesis in the peel was the result of cooperation among several genes, and debagging with reflective film mulching had better effect on promoting Ant accumulation than debagging with non-reflective film mulching.

Keywords: peach; coloration; reflective film; color difference; pigment; gene expression

1. Introduction

In the middle and lower reaches of the Yangtze River, the majority of late-maturing peach varieties often show poor color and visual appearance due to issues associated with the unfavorable climate, thus resulting in low consumer acceptance (Zhang et al., 2013). To solve these problems, breeders have focused in recent years on breeding strains with high coloring

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potential, good fruit quality, and strong pest and disease resistance. These efforts have led to several excellent strains including ‘Meishuai’ (Jia et al., 2005), ‘Shuanghongyan’ (Chu et al., 2010), ‘Ruiguang 39’ (Guo et al., 2011), and ‘Xiahui 8’ (Yu et al., 2014), which provide more choices for consumers.

Fruit bags, especially inner black bags, need to be removed before harvest so the fruits can have a better color. Reflective films can improve both the light distribution of the inner side of the tree canopy to increase the net photosynthetic rate (Zhou and Wang, 2009) and the fruit color (Layne et al., 2001, 2002). Using the reflective film to improve fruit quality has been widely reported (Miller and Greene, 2003; Iglesias and Alegre, 2009; Shi et al., 2011), but the mechanisms whereby the reflective film influences fruit color remains elusive.

In this study, we investigated how using the reflective film before harvest affects coloration physiology and gene expression of fruits using the late-maturing peach variety ‘Xiahui 8’ as a model. Our results provide insights into the understanding of how reflective films affect anthocyanin accumulation in peach skin, the mechanism regulating peach color formation, and the breeding of high-quality peach strains.

2. Materials and methods

2.1. Plant materials and treatments

The experiments were carried out in the Experimental Peach Orchard of Jiangsu Academy of Agricultural Sciences in 2014. Five-year-old ‘Xiahui 8’ peach trees of similar growth rate were selected as test materials, planted in north-south rows using Maotao as rootstocks, spaced at 3 m × 5 m, and managed according to the routine cultivation measures of the orchard.

Fruit thinning was conducted in late May so all trees had a consistent amount of fruits. After a whole garden spraying with insecticide and fungicide (1.8% 2 500× abamectin emulsifiable concentrate + 50% 600× carbendazim), fruits of similar size and developmental stages were bagged using double-layer bags (yellow outside and black inside double-layer bags) which were inflated and tied to seal. According to preliminary results obtained in 2013, bags were removed at four o’clock pm on the twelfth day before the harvest date. Then silver reflective films (2 m of width, 0.02 cm of thickness, purchased from Mikado Plastic Sheets Development Co., Ltd, Jiangsu, China) were laid on the orchard floor along the tree rows with the total film width no less than the width of crown projected area. This group of trees was referred to as the debagging with reflective film group. Meanwhile, a group of control trees that had their bags removed but received no reflective film treatment were referred to as the debagging with non-reflective film group, and another group of control trees that received neither fruit bag nor reflective film treatment was referred to as the non-bagging with non-reflective film group. Each group consisted of three subgroups of two trees each, thus representing three replicates. Trees that received reflective film treatment and trees that received no reflective film treatment were spaced by three trees to avoid light distribution interference. Three fruit samples were collected immediately after laying the reflective film and were labeled as samples twelve days before harvest. Thereafter, fruit samples were collected on the ninth, sixth, third and zero days before harvest. Each time 60 fruits were randomly picked from the middle to lower part of canopy, immediately placed in ice boxes, and transferred to the laboratory. After quick photograph and color measurement, skin slices with thickness of about 0.5 mm were carefully cut, quickly frozen in liquid nitrogen, and stored at a −70 ℃ refrigerator. For each treatment, three replicates were prepared.

2.2. Parameter measurement

2.2.1. Color measurement

Color intensity was measured using Color Quest XE colorimeter (Hunter Lab, Reston, VA, USA) at four symmetrical equatorial points. The lightness (L^*) and color-space coordinates (a^* and b^*) were recorded, and chroma (C), hue angle (h) and a^*/b^* were calculated as described (Voss, 1992; Koukounaras et al., 2009). The result was presented as the average of the four measured symmetrical equatorial points.

2.2.2. Pigment content

According to Zapsalis and Francis (1965), anthocyanin was extracted using 1% HCl-methanol in the dark, and then the concentration was calculated based on the absorbance values at the wavelength of 650, 620, and 530 nm. According to Lichtenthaler and Wellburn (1983), skin chlorophyll was extracted with 95% ethanol in the dark and the concentration was calculated based on the absorbance values at wavelengths of 665 and 649 nm.

2.2.3. Phenylalanine ammonia lyase (PAL) activity

According to Koukol and Conn (1961), skin extracts were centrifuged at 14 000 r · min^{-1} for 20 min and the supernatant was used to measure the absorbance value at the wavelength of 290 nm on a spectrophotometer. One unit of PAL activity was defined as a change of 0.01 optical density units per hour.

2.2.4. Total RNA extraction from peach skin and qRT-PCR

Total skin RNA was extracted using the improved CTAB method, RNA quality was examined using agarose gel electrophoresis, and cDNA was synthesized from total skin RNA using Prime Script RT reagent Kit with gDNA Eraser kit (TaKaRa). According to sequence information deposited in GenBank, primers were designed for the five genes *UFGT*, *DFR*, *LDOX*, *CHS*, and *F3H* (Guo et al., 2013), and the housekeeping gene *TEF2* was used as internal reference. Primers were then synthesized by Invitrogen (Table 1).

Table 1 Primers of real-time quantitative PCR

Gene name	Accession No.	Forward primer	Reverse primer	Product size/bp
PpUFGT	JX149550.1	CGTCAAACTACTCGCACGAC	GTGAAGTGCAGCTCGGCTAT	134
PpDFR	HM543571.1	TGAGAAACATGAGGCTGACG	TAACAGCCAACCGGAAAAAC	156
PpLDOX	EU292219	GACTGAGCCGCCAATCTTCC	TCAACAAAGCAGGTAGACAGTAGC	97
PpCHS	JN391444.1	ATCTCCGTGAAGTTGGGCTTACATTT	GTGTGCAATCCAGAATAGTGAGTTCCA	140
PpF3H	HM543570.1	GGAGCAACAATTGCAGGACT	CATCCACTGGCTAAGCACAT	154
TEF2		GGTGTGACGATGAAGAGTGATG	TGAAGGAGAGGGAAGGTGAAAG	

The qRT-PCR system consisted of 2 μL cDNA, 0.8 μL of upstream and downstream primer each, 10 μL reaction MIX, 6 μL ddH_2O, and 0.4 μL ROX Reference DyeⅡ. PCR reaction was initiated by a denaturation for 30 s at 95 ℃ followed by 40 cycles of 5 s at 95 ℃ and 34 s at 60 ℃. The expression level was presented as the fold change of expression $2^{-\Delta\Delta CT}$. Each result was the average of three replicates.

Excel was used for statistical analysis, SPSS 16.0 was used for significance analysis, and all of the data were tested by Duncan's new multiple range method.

3. Results

3.1. The effects of reflective film on color difference of peach skin

a^*/b^* value can be used to describe the true color of fruit, with a negative value indicating green color, a zero value indicating a color falling between green and yellow or orange-red, and a positive value indicating yellow or orange-red.

As shown in Fig. 1, on the twelfth day before harvest,

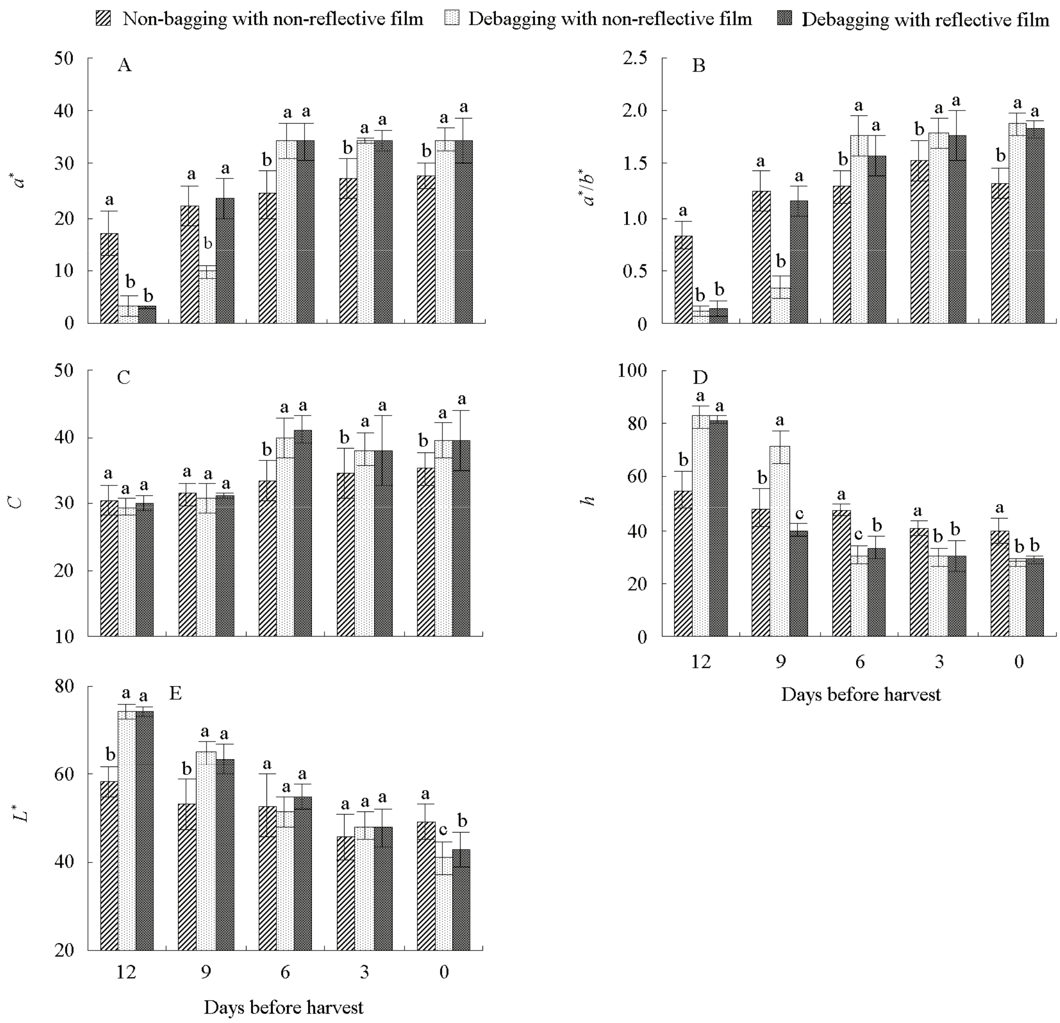

Fig. 1 Effect of reflective film mulching on peel color difference of 'Xiahui 8' peach

'Xiahui 8' peaches from the non-bagging with non-reflective film group, which received neither bag and nor reflective film treatment, had the highest a^* and a^*/b^* values and had the lowest h, L^* values. All of the treatments led to gradual increases in a^*, a^*/b^*, and C values, while leading to gradual decreases in h, L^* values, and these changes were most evident from the twelfth to the sixth day before the harvest.

From the sixth day to the day of the harvest, the fruits from the debagging with non-reflective film group, which had bags removed but received no reflective film treatment, and the debagging with reflective film group, which had bags removed and received reflective film treatment, both had significantly higher a^*, a^*/b^* and C values, but had lower h values than the non-bagging with non-reflective film group. The color difference of three groups increased variably with time. From the twelfth to the ninth day before the harvest, the debagging with reflective film group showed the highest increase in a^*/b^* values (an increase of 666.67%), the debagging with non-reflective film group increased less (183.33%), and the non-bagging with non-reflective film group increased the least (50.60%). But after that the initial increase, the debagging with reflective film group and the debagging with non-reflective film group showed no color difference. In contrast to color difference, from the twelfth to the ninth day before the harvest, h value of the debagging with reflective film group decreased the most, while the debagging with non-reflective film group decreased less, and the non-bagging with non-reflective film group decreased the least. From the third day to the day of the harvest, the debagging with reflective film group and the debagging with non-reflective film group showed similar h values. In addition, the C values of two treatment groups were quite similar during the test and were both significantly higher than that of the non-bagging with non-reflective film group from the sixth day to the day of the harvest.

As shown in Fig. 2, the fruit color change was consistent with the change in color difference, indicating that simple bag removal could significantly improve the red color of 'Xiahui 8' peaches and increase the brightness, but the further reflective film treatment can accelerate the red color formation.

3.2. *The effects of reflective film on pigment level of peach skin*

Anthocyanins level showed a gradual increase during the trial in all groups. Twelve days before harvest, two treatment groups showed significantly lower anthocyanin levels than the non-bagging with non-reflective film group (Fig. 3, A). However, the anthocyanin levels of the two treatment groups then showed rapid increases from the twelfth to the third day before the harvest, resulting in an 11.81-fold increase in the debagging with non-reflective film group and a 15.82-fold increase in the debagging with reflective film group, but during this time, the non-bagging with non-reflective film group only had a 4.65-fold increase in anthocyanin level.

Anthocyanin level showed a small increase from the third day to the day of the harvest, and the relative increase amount is the debagging with reflective film group > the debagging with non-reflective film group > the non-bagging with non-reflective film group ($P < 0.05$). Therefore, simple bag removal can accelerate the accumulation of anthocyanins in the skin of 'Xiahui 8' peaches, but further reflective film treatment can achieve even better results.

Fig. 2 Effect of reflective film mulching on fruit coloration of 'Xiahui 8' peach

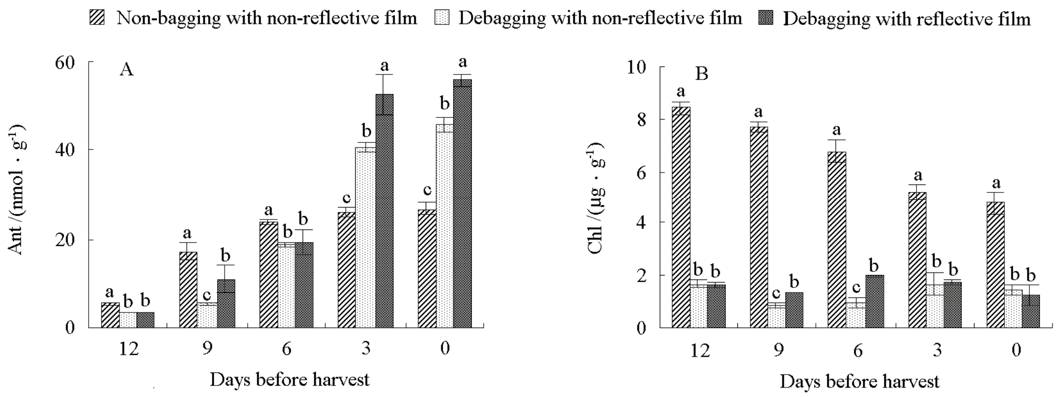

Fig. 3 Effect of reflective film mulching on peel pigment content of ‘Xiahui 8’ peach

Skin chlorophyll level showed different trends to anthocyanin level (Fig. 3, B). The non-bagging with non-reflective film group gradually decreased its chlorophyll level throughout the trial, but always had a higher chlorophyll level than the two treatment groups. At the harvest, the two treatment groups had similar chlorophyll level.

3.3. The effects of reflective film on phenylalanine ammonia lyase (PAL) activity in peach skin

As shown in Fig. 4, PAL activity kept decreasing in the non-bagging with non-reflective film group of ‘Xiahui 8’ peaches, while increasing at first and then decreasing in the debagging with non-reflective film group and the debagging with reflective film group. PAL activity reached peak between the ninth and the sixth day before the harvest in two treatment groups.

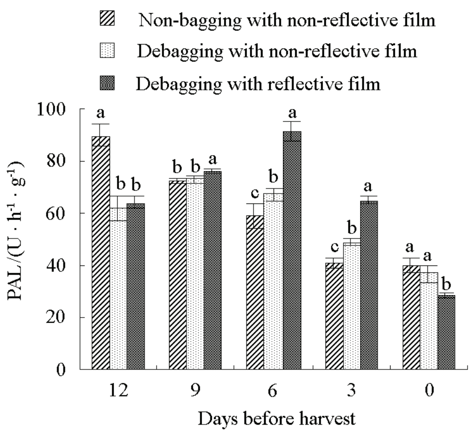

Fig. 4 Effect of reflective film mulching on peel PAL activity of ‘Xiahui 8’ peach

The non-bagging with non-reflective film group had the highest skin PAL activity twelve days before harvest, while the two treatment groups had similarly lower skin PAL activity. The relative PAL activity from the sixth to the third day before harvest is the debagging with reflective film group > the debagging with non-reflective film group > the non-bagging with non-reflective film group ($P < 0.05$). While at harvest, the debagging with reflective film group had the lowest PAL activity. Thus the two treatment groups showed different trends in terms of PAL activity change, indicating that the light condition significantly affected PAL activity.

3.4. The effects of reflective film on the expression of genes associated with anthocyanin synthesis in peach skin

Debagging with reflective film and debagging with non-reflective film treatment both affected the expression of genes related to anthocyanin synthesis in the skin of ‘Xiahui 8’ peach fruits.

Twelve days before harvest, the expression of *UFGT* and *CHS* was significantly higher in the two treatment groups than in the non-bagging with non-reflective film group. A short time of exposure to natural light significantly promoted the transcription of these genes, with the debagging with reflective film group showing the highest expression level, followed by the debagging with non-reflective film group. During this trial, *UFGT* expression gradually increased and reached the highest level three days before harvest in the non-bagging with non-reflective film group, and *CHS* expression showed the same change as *UFGT*. At harvest, the relative *CHS* expression level was the non-bagging with non-reflective film group > the debagging with non-reflective film group > the debagging with reflective film group (Fig. 5, A, D). In comparison to the non-bagging with non-reflective film group, the two treatment groups both showed a delayed expression of *DFR*, *LDOX*, and *F3H*. The expression levels of the three genes were low twelve days before harvest, but increased with time. But as the trials progressed, the expression of the three genes began to decrease in the two treatment groups. Interestingly, the expression levels of *LDOX* and *F3H* rose again during the last three days of the trial in the debagging with reflective film group. In addition,

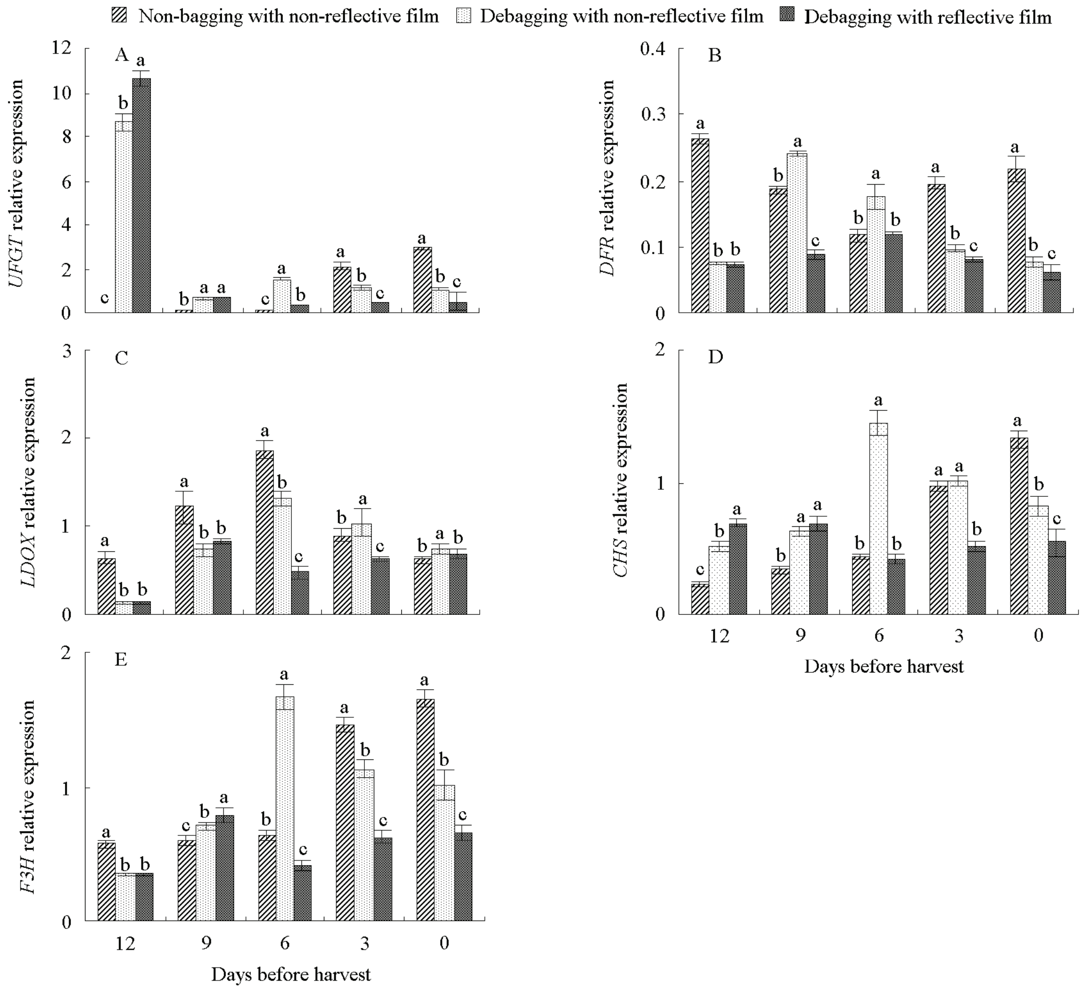

Fig. 5 Effect of reflective film mulching on peel relative expression of anthocyanin structural genes of ‘Xiahui 8’ peach

from the sixth day to the day of the harvest, the expression levels of the three genes were significantly higher in the debagging with non-reflective film group than in the debagging with reflective film group. The expression of the three genes show different patterns in the non-bagging with non-reflective film group, as the peak level of *DFR*, *LDOX*, and *F3H* appeared on the twelfth day, sixth day and the zero day before harvest, respectively (Fig. 5, B, C, E). Taken together, these results suggest that there may be differences in color formation between the debagging with non-reflective film group and the debagging with reflective film group, and there also may be differences in the mechanism of fruit anthocyanin accumulation between the two treatment groups and the non-bagging with non-reflective film group.

4. Discussion

Light is the driving force for photosynthesis, and also the leading environmental factor affecting growth and development of plants. Bagging first affects the level and quality of light the fruits receive (Ma et al., 2014). The light transmittance rate of the double-layer fruit bags used in this trial is so low that the fruits almost received no natural light, resulting in inhibition of anthocyanins and chlorophyll synthesis and a very different fruit color from the non-bagging with non-reflective film group which is consistent with the results of Ma et al. (2012). When the double-layer bags were removed, the fruits were suddenly shifted from a dark environment to a full light environment, local temperature and humidity of fruits also changed, and the use of a reflective film further increased the light distribution inside the tree canopy. All these changes led to different orchard microenvironment (Tarara et al., 2000), which increased light absorption by the canopy and fruits and the fruit surface temperature (Green et al., 1995). The level of anthocyanin and the ratio of anthocyanin/chlorophyll together determined the skin color difference, especially the red color-associated parameter a^*, a^*/b^* and h. In this test, the a^* and a^*/b^* of the two treatment groups already reached relatively high levels six days before harvest, while their *h* value decreased. Red color appeared more quickly in the debagging with reflective film group than in the debagging with non-reflective film group; nevertheless, the two groups both showed better red color and increased brightness than the non-bagging with non-reflective film group, consistent with previous findings (Andris and Crisosto, 1996; Ju et al., 1999; Glenn and Puterka, 2007).

Biosynthesis of anthocyanins is a light-dependent process (Saure, 1990). Reflective films laid under the trees increase the light-receiving area and the light intensity of the fruit from different angles to extend the light period, which contribute to anthocyanin formation and accumulation (Ju et al., 1991). Reflective films act mainly in two ways to promote anthocyanin accumulation: First, reflective films increase canopy photosynthesis and fruit carbon assimilation, indirectly promoting the synthesis of anthocyanins (Lancaster and Dougall, 1992; Williams et al., 1993); Second, reflective films increase the PAL activity and upregulate the expression of related genes including *UFGalT* to promote anthocyanin synthesis (Ju et al., 1995). In this study, the rise in PAL activity in peach skin was more significant in the debagging with reflective film group than in the debagging with non-reflective film group, which was accompanied by a quicker and more significant appearance of red color in the debagging with reflective film group than in the debagging with non-reflective film group. Accumulation of anthocyanins ultimately depends on the expression of related genes. The related genes *UFGT*, *DFR*, *LDOX*, *CHS*, and *F3H* have been studied in grapes (Kobayashi et al., 2002; Zhang et al., 2014) and apples (Ju et al., 1995; Kondo et al., 2002). Additionally, the anthocyanin biosynthesis pathway has also been relatively well understood. However, the molecular mechanism of how the reflective film controls anthocyanin accumulation in peach skin has never been investigated. In this study, different genes showed very different responses when the fruits were suddenly exposed to natural light. The expression of *UFGT* and *CHS* was higher in the two treatment groups than in the non-bagging with non-reflective film group. In the initial trial, *UFGT* expression was downregulated. Although the expression levels of *DFR*, *LDOX*, and *F3H*, were upregulated in the initial trial, they were lower in the two treatment groups than in the non-bagging with non-reflective film group. Moreover, anthocyanin accumulation was faster in the two treatment groups than in the non-bagging with non-reflective film group. Taken together, these results suggest that anthocyanin synthesis in the skin of 'Xiahui 8' peaches requires coordination of many genes. The expression levels of *UFGT* and *CHS* reached peak levels twelve days before harvest and later decreased in the debagging with reflective film group. Thus, these expression results are not consistent with the results of anthocyanin accumulation; one possibility is that early high expression levels of the two genes may produce enough enzymes to maintain anthocyanin synthesis even in later times when their expressions were downregulated. Synthesis of large amount of anthocyanins may inhibit the expression of the upstream genes in a negative feedback, resulting in a negative association between gene expression and anthocyanin level (Guo et al., 2013). Similarly, the expression level of *DFR* from the twelfth to the sixth day, and the expression levels of *LDOX* and *F3H* from the twelfth to ninth day, before harvest are also not positively associated with the accumulation of anthocyanins in the debagging with reflective film group. The fruits from the debagging with non-reflective film group and the debagging with reflective film group received different intensities of light, and the two groups showed different expression levels of anthocyanin biosynthesis-related genes. The expression levels of *DFR*, *LDOX*, *CHS*, and *F3H* were upregulated at first and then downregulated, suggesting that an early upregulation of these genes promoted the accumulation of anthocyanins in early and later trials, consistent with previous studies (Zhang et al., 2013, 2014).

In summary, in comparison to the natural growth conditions, bagging and bagging plus reflective film both can significantly improve the red color of the fruits, increase anthocyanin levels, and promote the transcription of anthocyanin synthesis-related genes, with bagging plus reflective film showing the best results.

Acknowledgements

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